REMARKS

Claims 1-20 are pending in this application. Reconsideration of the application is requested in view of the following remarks:

Rejection under 35 U.S.C. § 103(a)

Claims 1-20 are rejected as being unpatentable over Sager et al. (U.S. Patent No. 5,470,970) in view of the abstracts of Petrovich et al. (Radiology 144(4): 905-908, 1982), Weber et al. (Otolaryngology – Head and Neck Surgery 99(1): 16-23, 1988), Tytor et al. (Clinical Otolaryngology 15(3): 235-252, 1990), Eiband et al. (American Journal of Surgery 158(4): 314-317, 1989), Huwer et al. (European Journal of Cardio-Thoracic Surgery 6(9): 498-502, 1992), Nagel et al. (Zentralblatt fur Chirurgie 119(4): 225-232, 1994), and van der Velden et al. (Cancer 75(12): 2885-2890, 1995). Applicants respectfully traverse.

Claims 6 and 16 will be discussed first. Claim 6 is drawn to a method of determining the relative probability of survival for a subject with squamous cell carcinoma; claim 16 is drawn to a method of determining whether a subject with squamous cell carcinoma has a lymph node containing cancerous cells. Both methods involve two steps: (1) determining the level of maspin gene expression in a biological sample from a subject with squamous cell carcinoma, and (2) comparing the level with a threshold level of maspin gene expression. If the level of maspin gene expression in the biological sample is below the threshold level, it indicates that the subject has a relatively low probability of survival or a lymph node containing cancerous cells.

The Examiner asserted that the primary reference, Sager et al., teaches a method of staging a carcinoma according to the level of maspin gene expression, in which a lower expression level is indicative of a later stage. On the other hand, the Examiner acknowledged that Sager et al. does not teach correlation between the level of maspin gene expression and the probability of survival or the presence of cancerous lymph nodes. The Examiner then pointed out that the secondary references, as listed above, teach correlation between the stage of cancer and the probability of survival or the presence of cancerous lymph nodes in patients with squamous cell carcinoma. It is the Examiner's conclusion that it would have been prima facie obvious to a skilled artisan to extend the correlation between a lower maspin expression level

and a later cancer stage to a correlation between a lower maspin expression level and a lower probability of survival or the presence of cancerous lymph nodes, and that a skilled artisan would have been motivated to do so with a reasonable expectation of success.

Applicants disagree. The method of Sager et al. is based on their discovery that maspin is expressed in normal mammary epithelial cells, and that the level of maspin expression decreases during progression to breast cancer. See, e.g., column 3, lines 2-13. There is no indication in Sager et al. that maspin is also expressed in normal squamous cells, nor that the level of maspin expression decreases during progression to squamous cell carcinoma. None of the secondary references mentions maspin gene expression in squamous cells or its down-regulation during squamous cell carcinoma development. Note that cancers of epithelial cells are classified into many different types according to their origins. See, e.g., page 242, Pathologic Basis of Disease, 4th edition. Edited by Ramzi S. Cotran, Vinay Kumar, and Stanley L. Robbins. 1989. W. B. Saunders Company, Harcourt Brace Jovanovich, Inc., Philadelphia, PA, a copy of which is attached hereto as "Exhibit A." Although all of these cancers derive from epithelial cells, they are histologically and morphologically distinct from one another and have different cytogenetic abnormalities. The fact that one gene is expressed in one type of epithelial cells does not necessarily imply that it is also expressed in another type of epithelial cells. Further, even if the gene is expressed in both types of epithelial cells, it may or may not be expressed in the same manner during cancer development. Indeed, maspin has been found to be down-regulated in breast and prostate carcinomas but up-regulated in pancreatic and ovarian carcinomas. Over-expression of maspin in ovarian carcinoma is indicative of high tumor grade and short survival. See, e.g., Sood et al., Clinical Cancer Research 8: 2924-2932, 2002, a copy of which is attached hereto as "Exhibit B." Since none of the references cited by the Examiner, alone or combined, discloses maspin gene expression in squamous cells or decreased maspin gene expression during squamous cell carcinoma development, a skilled artisan would have no motivation to apply the staging method of Sager et al. for breast cancer to squamous cell carcinoma, let alone motivation to extend the staging method to a method of predicting the relative probability of survival or the presence of cancerous lymph nodes in subjects with squamous cell carcinoma. Moreover, maspin gene expression may differ in various types of epithelial cells. Thus, even if a skilled artisan would have been motivated to combine the primary and secondary references to come up

with the method of predicting the relative probability of survival or the presence of cancerous lymph nodes in subjects with squamous cell carcinoma according to the level of maspin gene expression, there would have been no reasonable expectation of success for such a method without the evidence provided by Applicants (see, e.g., Table 1 at page 9 of the specification and Fig. 1).

For the reasons set forth above, claims 6 and 16 are patentably distinguishable over the cited art. By the same token, claims 1 and 11 are also patentable. So are their respective dependent claims, i.e., claims 2-5 dependent from claim 1, claims 7-10 dependent from claim 6, claims 12-15 dependent from claim 11, and claims 17-20 dependent from claim 16.

CONCLUSION

Applicants submit that the ground for rejection asserted by the Examiner has been overcome, and that claims 1-20, as pending, define subject matter that is non-obvious and thus patentable. On this basis, it is submitted that allowance of this application is proper, and early favorable action is solicited.

EXHIBIT A

Table 6-1. *Nomenclature of Tumors*

TISSUE OF ORIGIN	BENIGN	MALIGNANT
I. Composed of one parenchymal cell type		
A. Tumors of mesenchymal origin		***Sarcomas***
(1) Connective tissue and derivatives	Fibroma	Fibrosarcoma
	Myxoma	Myxosarcoma
	Lipoma	Liposarcoma
	Chondroma	Chondrosarcoma
	Osteoma	Osteogenic sarcoma
(2) Endothelial and related tissues		
Blood vessels	Hemangioma Capillary Cavernous	Angiosarcoma
Lymph vessels	Lymphangioma	Lymphangiosarcoma
Synovia		Synovioma (synoviosarcoma)
Mesothelium (lining cells of body cavities)		Mesothelioma
Brain coverings	Meningioma	Invasive meningioma
Glomus	Glomus tumor	
(3) Blood cells and related cells		
Hematopoietic cells		Myelogenous leukemia Monocytic leukemia
Lymphoid tissue		Malignant lymphomas Lymphocytic leukemia Plasmacytoma (multiple myeloma)
Langerhans' cells		Histiocytosis X
Monocyte-macrophage		? Histiocytic lymphoma ? Hodgkin's disease
(4) Muscle		
Smooth muscle	Leiomyoma	Leiomyosarcoma
Striated	Rhabdomyoma	Rhabdomyosarcoma
B. Tumors of epithelial origin		***Carcinomas***
(1) Stratified squamous	Squamous cell papilloma	Squamous cell or epidermoid carcinoma
(2) Basal cells of skin or adnexa		Basal cell carcinoma
(3) Skin adnexal glands		
Sweat glands	Sweat gland adenoma	Sweat gland carcinoma
Sebaceous glands	Sebaceous gland adenoma	Sebaceous gland carcinoma
(4) Epithelial lining		
Glands or ducts—well-differentiated group	Adenoma Papilloma Papillary adenoma Cystadenoma	Adenocarcinoma Papillary carcinoma Papillary adenocarcinoma Cystadenocarcinoma
Poorly differentiated group		Medullary carcinoma Undifferentiated carcinoma (simplex)
(5) Respiratory passages		Bronchogenic carcinoma Bronchial "adenoma"
(6) Neuroectoderm	Nevus	Melanoma (melanocarcinoma)
(7) Renal epithelium	Renal tubular adenoma	Renal cell carcinoma (hypernephroma)
(8) Liver cells	Liver cell adenoma	Hepatoma (hepatocellular carcinoma)
(9) Bile duct	Bile duct adenoma	Bile duct carcinoma (cholangiocarcinoma)
(10) Urinary tract epithelium (transitional)	Transitional cell papilloma	Papillary carcinoma Transitional cell carcinoma Squamous cell carcinoma
(11) Placental epithelium	Hydatidiform mole	Choriocarcinoma
(12) Testicular epithelium (germ cells)		Seminoma Embryonal carcinoma
II. More than one neoplastic cell type—mixed tumors—usually derived from one germ layer		
(1) Salivary glands	Pleomorphic adenoma (mixed tumor of salivary gland origin)	Malignant mixed tumor of salivary gland origin
(2) Renal anlage		Wilms' tumor
III. More than one neoplastic cell type derived from more than one germ layer—teratogenous		
(1) Totipotential cells in gonads or in embryonic rests	Mature teratoma, dermoid cyst	Immature teratoma

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The Paradoxical Expression of *Maspin* in Ovarian Carcinoma[1]

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ABSTRACT

Maspin is a noninhibitory member of the serpin family that is down-regulated in breast carcinoma but overexpressed in pancreatic carcinoma. There are no published data regarding the role of maspin in ovarian carcinoma, which is the focus of the present study. Ovarian cell lines (normal and cancer) and tumors (80 invasive, 14 benign, and 10 low malignant potential) were evaluated for maspin expression and localization. Normal ovarian surface epithelial cells had low levels of maspin. Two of four ovarian cancer cell lines (OVCAR3 and SKOV3) expressed maspin, whereas the cell line EG had weak expression, and 222 had no detectable maspin. Subcellular fractionation studies revealed that the two maspin-positive ovarian cancer cell lines contained maspin in both the nuclear and cytosolic compartments. Wild-type maspin was transfected into the aggressive ovarian cancer cell lines SKOV3 and 222. The *in vitro* invasive activity of the maspin-transfected cell lines was 44–68% lower than respective controls. The histopathology analysis revealed that among the ovarian tumors examined, 57 (71%) were ranked positive for maspin. Thirty (37%) of the invasive tumors overexpressed maspin. Invasive cancers were more likely to have predominantly cytoplasmic staining compared with benign and low-malignant-potential tumors. Maspin overexpression was significantly associated with a high tumor grade ($P = 0.004$), the presence of ascites ($P = 0.02$), a lower likelihood of optimal surgical cytoreduction ($P = 0.04$), and a shorter duration of overall survival (median survival, 6.33 *versus* 2.67 years; $P = 0.003$). The Cox proportional hazards multivariate model revealed that maspin overexpression and high stage were independent predictors of survival. Thus, maspin was found to be overexpressed in a substantial proportion of ovarian tumors, which may serve as an adverse prognostic factor; however, its localization may provide new clues as to its activity and function. These paradoxical results may offer new insights regarding the role of maspin in ovarian cancer progression that may also impact diagnosis and treatment strategies.

INTRODUCTION

Maspin (mammary serpin) is a serine protease inhibitor member of the serpin family (1). The *maspin* gene was originally identified in normal mammary epithelium by subtractive hybridization on the basis of its expression at the mRNA level (1). It has been shown to have tumor suppressive activity attributable to the inhibition of breast cancer cell motility, invasion, and metastasis (2–4). Maspin encodes a 375-amino-acid protein (M_r of 42,000) with sequence homology to other inhibitory serpins (2, 5). The reactive site loop, situated near the COOH terminus, is one of the highly variable regions and is the domain that binds to the active site of the serine protease. The *maspin* gene is part of a serpin locus cluster at chromosome 18q21.3-q23. Several important transcription factor-binding sites are present within the 1-kb promoter region, namely Ets, Ap1, HRE, and p53 (6). Studies have revealed that maspin is largely an intracellular protein that is soluble in the cytoplasm and is also found associated with secretory vesicles.[3] Maspin is located at the cell membrane interface with the extracellular matrix and does not act as a classical inhibitory serpin with antiprotease activity against trypsin-like proteases (7–9). Rather, small amounts of maspin are secreted but appear to remain associated with distinct structures at the cell surface.

Maspin appears to play a critical role in normal embryological development.[4] The homozygous *maspin* knockout mice have been shown to produce an embryonic lethal.[4] Interestingly, the existing knockout mice for other serpins and their protease targets are all viable.[4] Female mice heterozygous for *maspin* are less likely to get pregnant, and, if they do, they deliver a smaller litter size compared with the wild-type mice.[4] There are effects on normal ovarian function as well: ovulation efficiency of

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[3] Pemberton, P. A. Maspin: functional insights from a structural perspective. *In:* M. J. C. Hendrix (ed.), Maspin. Internet address: www.landesbioscience.com.
[4] Zhang, M. The role of maspin in tumor progression and normal development. *In:* M. J. C. Hendrix (ed.), Maspin. Internet address: www.landesbioscience.com.

maspin heterozygotes is greatly reduced compared with controls.

Although, at present, the molecular and biological mechanisms of the function(s) of maspin remains largely unknown, there is evidence that maspin interacts with the p53 tumor suppressor pathway and may function as an inhibitor of angiogenesis *in vitro* and *in vivo* (footnote[4] and Ref. 10). Pemberton *et al.* (11) have demonstrated the presence of maspin in the epithelium of several normal human organs (such as prostate, thymus, testis, small intestine, and colon) and particularly in the myoepithelium of breast tissue, in which it is predominant and probably functions both intra- and extracellularly. However, maspin was not detected in the normal ovary in that study.

Maspin is expressed in normal human mammary and prostate epithelial cells but is down-regulated during cancer progression (1, 12, 13). The loss of *maspin* gene expression with increasing malignancy is regulated at the transcriptional level (6). Recent studies have reported on the role of cytosine methylation and chromatin condensation in the down-regulation of maspin expression during neoplastic progression (14). However, other findings by Maass *et al.* (15) showed that maspin was overexpressed in pancreatic cancers. Specifically, 5 of 9 pancreatic cancer cell lines had maspin expression along with 23 of 24 tumor specimens. Maspin expression was not detected in normal pancreatic tissues, and patient outcome was not assessed in this study (15). To date, there have been no published reports evaluating the role of maspin in ovarian carcinomas, particularly with respect to clinical outcome. Thus, we undertook the present study with the following aims: (*a*) evaluate the clinical relevance of maspin expression in ovarian cancer, and; (*b*) evaluate the role of maspin in ovarian cancer invasion in experimental models.

MATERIALS AND METHODS

Cell Culture. The ovarian cancer cell lines used in this study were OVCAR3, SKOV3, EG, and 222. The derivation and sources of these cell lines have been reported previously (16). These cells were maintained and propagated *in vitro* by serial passage in RPMI 1640 supplemented with 15% fetal bovine serum and 0.1% gentamicin sulfate (Gemini Bioproducts; Calabasas, CA). The immortalized normal human ovarian surface epithelial cell line HIO 180 was a kind gift from Dr. Andrew Godwin at the Fox Chase Cancer Center, Philadelphia, PA. The immortalized normal breast epithelial cell line 1436N1 was a kind gift from Dr. Shijie Sheng at Wayne State University, Detroit, MI (16). These lines were maintained in Medium 199/MCDB 105 supplemented with 15% fetal bovine serum and 0.1% gentamicin sulfate. All of the cell lines are routinely screened for *Mycoplasma* species (GenProbe detection kit, Fisher, Itasca, IL). All of the experiments were performed with 70–80% confluent cultures.

Invasion Assay. The Membrane Invasion Culture system (MICS) chamber was used to measure the *in vitro* invasiveness of all of the cell lines used in this study (17, 18). Briefly, a polycarbonate membrane with 10 μM pores (Osmonics; Livermore, CA) was uniformly coated with a defined basement membrane matrix consisting of human laminin/type IV collagen/gelatin and used as the intervening barrier to invasion. Both upper and lower wells of the chamber were filled with serum-free RPMI containing 1× MITO+ serum supplement (Collaborative Biomedical; Bedford, MA). Single-cell tumor suspensions were seeded into the upper wells at a concentration of 1×10^5 cells/well. After a 24-h incubation in a humidified incubator at 37°C with 5% CO_2, cells that had invaded through the basement membrane were collected, stained, and counted by light microscopy (18). For chemoinvasion assays, conditioned medium from normal skin fibroblasts (kindly provided by Dr. Gregory Goldberg, Washington University, St. Louis, MO) was added to the lower wells. Invasiveness was calculated as the percentage of cells that had successfully invaded through the matrix-coated membrane to the lower wells compared with the total number of cells seeded into the upper wells and corrected for cell proliferation.

Formalin-fixed, Paraffin-embedded Samples for Maspin Immunohistochemical Staining. All of the samples were collected in compliance with requirements of the Institutional Review Board for the Protection of Human Subjects. Formalin-fixed, paraffin-embedded samples were sectioned at 4 μm and stained with H&E for identification. Sections adjacent to the H&E-stained sections were used for immunohistochemical staining. All of the slides were deparaffinized using xylene, 100% ethanol, and 95% ethanol, followed by a thorough deionized water wash. A water bath antigen-recovery technique using citrate buffer (pH 6.0) was performed on all of the slides. The immunohistochemical staining for maspin was performed on the Dako Autostainer using the Vectastain Universal Elite ABC, Peroxidase kit (Vector Laboratories, Inc., Burlingame, CA) to detect mouse antihuman maspin (dilution 1:200; PharMingen, San Diego, CA). After deparaffinization and antigen recovery, slides were washed in TBST.[5] Three blocking steps were applied: 0.03% hydrogen peroxide (Dako) for 15 min followed by a TBST wash, avidin and biotin blocks were applied for 15 min in each solution followed by a TBST wash after each step, and finally the Protein Block Serum-Free (Dako) was applied for 15 min. The primary antibody was applied to the slides and incubated for 60 min; slides were rinsed in TBST, followed by application of the vectastain secondary for 20 min. This procedure was followed by a TBST wash and then incubation with the ABC Reagent for 20 min. Color was produced by using DAB+ (brown) substrate (Dako) for 3 min. Slides were counterstained with Mayer's hematoxylin for 5–10 min.

All of the samples were reviewed by a board-certified pathologist (M. S. F.) who was blinded to the clinical outcome of these patients. Maspin expression was determined by assessing semiquantitatively the percentage of stained tumor cells and the staining intensity. The percentage of positive cells was rated as follows: 0 points, 0–5%; 2 points, 6–50%; 3 points, >50%. The staining intensity was rated as follows: 1 point, weak intensity; 2 points, moderate intensity; 3 points, strong intensity. Points for expression and percentage of positive cells were

[5] The abbreviations used are: TBST, Tris-buffered saline and Tween 20; OMS, overall maspin score; RT-PCR, reverse transcription-PCR; LMP, low malignant potential; HRE, hormonal responsive element; GAPDH, glyceralde hyde-3-phosphate dehydrogenase.

added, and an OMS (0–3) was assigned. Tumors were categorized into four groups: negative (OMS = 0), ≤ 5% cell stained, regardless of intensity; weak expression (OMS = 1), 2–3 points; moderate expression (OMS = 2), 4–5 points; and strong expression (OMS = 3), 6–7 points.

Subcellular Fractionation Studies. Cells were harvested and cell pellets used to prepare cytosolic and nuclear fractions. The cell pellet was suspended in buffer A [10 mM HEPES buffer (pH 7.9), containing 10 mM NaCl, 1 mM DTT, 10% glycerol, 15 mM $MgCl_2$, 0.2 mM EDTA, 0.1%NP40, protease inhibitor cocktail, and 1 mM phenylmethylsulfonyl fluoride] and subjected to three cycles of freeze-thaw and centrifuged at 4500 × *g* for 10 min. The supernatant (cytosolic fraction) was removed, the pellet washed twice with buffer A, resuspended in buffer B (buffer A containing 500 mM NaCl) and left on ice for 30 min with occasional vortexing. The mixture was centrifuged at 25,000 × *g* for 20 min to yield the nuclear fraction. The protein content of each fraction was determined using a bicinchoninic acid protein assay reagent kit (Pierce Corporation, Rockford, IL).

Maspin Transfection. Cells were plated at 5×10^5 cells/well on 6-well dishes. The cell lines 222 and SKOV3 were transfected with 2 μg of wild-type *maspin* cDNA in pcDNA3.1 vector (Invitrogen Corporation, Carlsbad, CA) using LipofectAMINE 2000 Reagent (Invitrogen), following the manufacturer's protocol. Forty-eight h after the transfection, G418-containing medium was added and changed every 48–72 h thereafter. Sham constructs were created by transfecting pcDNA3.1 alone into both cell lines.

Western Blot Analysis. Cells were lysed with 1× RIPA buffer (50 mM Tris, 150 mM NaCl, 1% Triton X-100, and 0.5% deoxycholate) containing 25 μg/ml leupeptin (Sigma Chemical Co., St. Louis, MO), 10 μg/ml aprotinin (Sigma Chemical Co.), 1 mM sodium orthovanadate, and 2 mM EDTA. Cells were removed from the dishes by cell scraping. The samples were then subjected to three cycles of freeze-thaw and centrifuged at 12,500 rpm for 30 min. The protein concentration of the samples was determined using a bicinchoninic acid Protein Assay Reagent kit, and whole cell lysates were analyzed by 10% SDS-PAGE and stained with Coomassie BBR-250 (Sigma Chemical Co.) to ensure equal loading (data not shown). Samples were transferred to nitrocellulose (Schleicher and Schuell, Keene, NH). Blots were blocked with 5% nonfat milk for 1 h at room temperature. Blots were incubated with the monoclonal maspin antibody (1:500 dilution; PharMingen) for 1 h at room temperature with agitation, followed by incubation with a horseradish peroxidase-conjugated antimouse secondary antibody (1:5000; The Jackson Laboratory, Bar Harbor, ME). Blots were developed using an enhanced chemiluminescence detection kit (ECL; Amersham Pharmacia Biotech, Piscataway, NJ). For subcellular fractionation analyses, monoclonal actin (1:1500, Chemicon International, Temecula, CA) and monoclonal lamin B1 (1:500; Zymed, San Francisco, CA) antibodies were used.

RT-PCR Analysis. Total RNA was extracted from cells using a monophasic solution of phenol and guanidine isothiocyanate (Trizol reagent; Invitrogen). Total RNA (1 μg) was reverse transcribed using an oligo(dT) primer and M-MLV reverse transcriptase (Life Technologies, Inc., Rockville, MD) in a final reaction volume of 20 μl (60 min at 42°C). The reaction was stopped by heating at 95°C for 5 min. The resulting cDNA was amplified by PCR in GeneAmp 10× PCR buffer (Perkin-Elmer, Branchburg, NJ) with 20 pmol of gene-specific 3′ and 5′ primers, 2 units of Taq DNA polymerase in a total volume of 50 μl. Reactions were carried out for 30 cycles at 94°C for 1 min, 60°C for 2.5 min, and 72°C for 1 min in an Infinity Robocycler (Stratagene, La Jolla, CA). The following primers were used for maspin detection: forward, 5′-CCACAGGCTTGGAGAAGATTGA-3′; reverse, 5′-GGTCAGCATTCAATTCATCCTTGT-3′.

After RT-PCR, the reaction products were separated by electrophoresis on a 1% agarose gel. Gels were stained with ethidium bromide to visualize the PCR product size. To control for variance in loading and in PCR, samples were compared with GAPDH PCR products.

Northern Blot Analysis. The RNA extracted as above was separated by gel electrophoresis in 1% agarose containing 2.2 M formaldehyde. The gels were capillary blotted onto GeneScreen Plus nylon membranes (NEN/DuPont, Boston, MA), baked, and UV cross-linked. The membranes were then probed with radiolabeled cDNA probe. These blots were then reprobed with an actin cDNA to ensure equal loading.

Clinicopathological Variable Analysis. All of the patients underwent surgical exploration and cytoreduction as the initial treatment. The treating gynecologic oncologist determined the adjuvant therapy. Diagnosis was verified by a pathology review at the institutional gynecologic oncology tumor board. All of the patients were staged according to the International Federation of Gynecology and Obstetrics (FIGO) surgical staging system. A gynecologic pathologist reviewed the pathology for all patients.

Statistical Analysis. Either the χ^2 test or Fisher's exact test was used as appropriate to determine differences between variables using SPSS (SPSS Inc., Chicago, IL). Kaplan-Meier survival plots were generated and comparisons between survival curves were made with the log-rank statistic. The Cox proportional hazards model was used for multivariate analysis. A P of <0.05 was considered statistically significant.

RESULTS

Maspin Expression in Ovarian Cell Lines. Maspin expression in selected ovarian cell lines was assessed by RT-PCR, Western blot, Northern blot, and immunohistochemistry (Fig. 1*A–E*). The cell line 1436N1 is a normal breast epithelial cell line and was used as a positive control. The cell line HIO-180 is known to be poorly invasive (17) and demonstrated minimal maspin expression by Western blot and immunohistochemistry; and no expression was noted by RT-PCR and Northern blot (Fig. 1). Immunohistochemistry revealed that the maspin was mostly nuclear in location (Fig. 1*C*). Among the cancer cell lines, OVCAR3, SKOV3, and EG are known to be moderately invasive and cell line 222 is highly invasive in an *in vitro* invasion assay (17). The cell lines OVCAR3 and SKOV3 both expressed maspin, detected by Western blot, RT-PCR, and immunohistochemistry (Fig. 1, *A*, *C*, and *E*). Northern blot analysis revealed that maspin was expressed in OVCAR3, and a weaker band was seen for SKOV3 (Fig. 1*E*). Subcellular fractionation studies revealed that maspin was present in both the

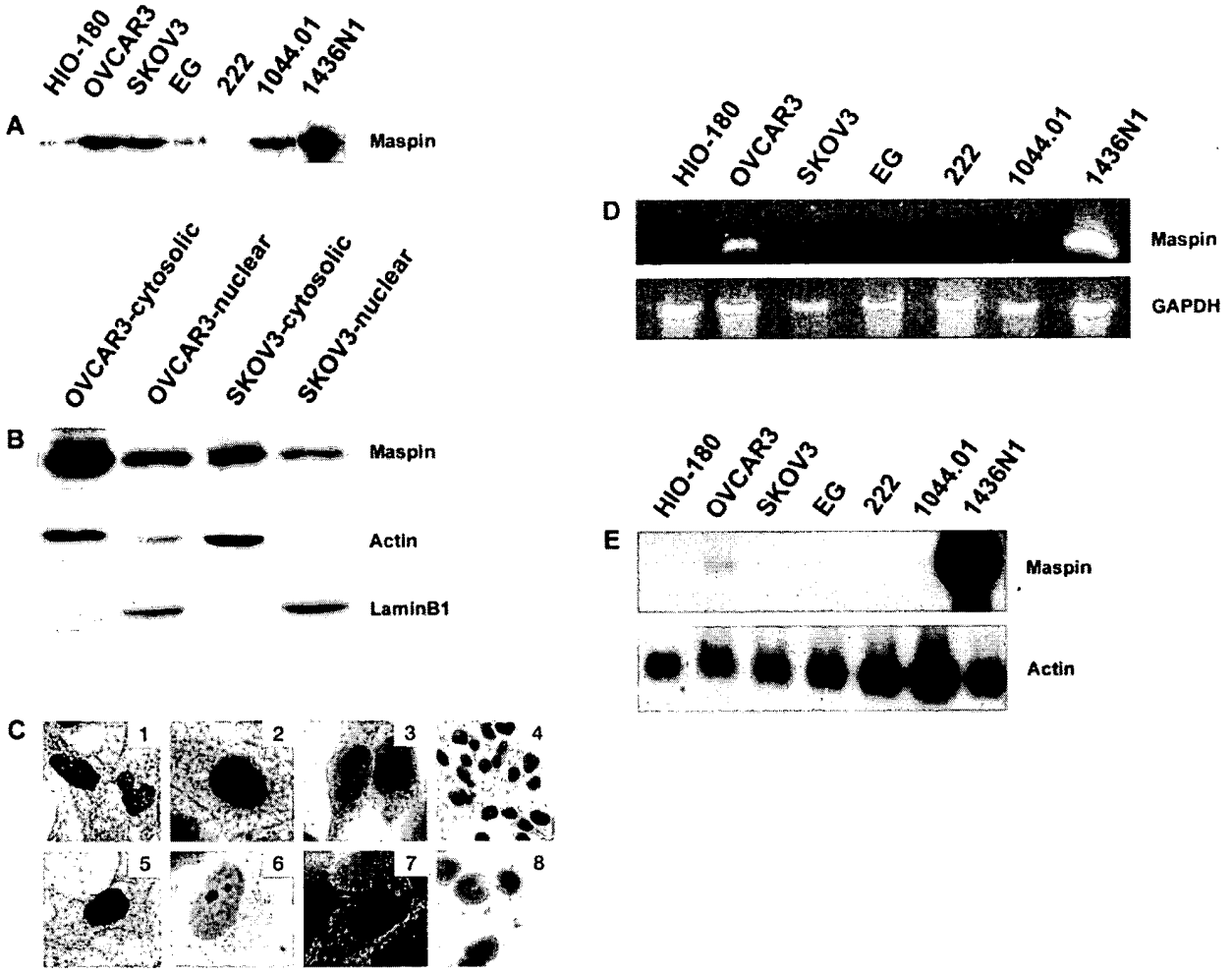

Fig. 1 Maspin analysis in normal ovarian surface epithelial cells (*HIO-180*), ovarian cancer cell lines (*OVCAR3*, *SKOV3*, *EG*, and *222*), and a high-stage tumor (*1044.01*). The cell line 1436N1 is a normal breast epithelial cell line and was used as a positive control. Maspin was analyzed by: (*A*) Western blot of whole cell lysates; (*B*) Western blot of subcellular fractions; (*C, 1–8*) immunohistochemical peroxidase staining for maspin in: (*1*) HIO-180, (*2*) OVCAR3, (*3–4*) SKOV3, (*5*) EG, (*6*) 222, (*7*) 1436N1, and (*8*) negative control; (*D*) RT-PCR; and (*E*) Northern blot. *C, 1–3* and *5–8*, ×630; *C, 4*, ×400.

nuclear and cytosolic fractions of OVCAR3 and SKOV3 (Fig. 1*B*). However, both immunohistochemistry and subcellular fractionation studies showed that most of the maspin was cytoplasmic in location. Monoclonal antibodies for actin and laminB1 were used as markers of cytosolic and nuclear fractions respectively (Fig. 1*B*). Full-length sequencing of the *maspin* gene was performed in OVCAR3 and SKOV3 to evaluate for a potential mutation. The following variations in sequence were noted: OVCAR3 [C*T*A→*G*TA (Leu→Val), nucleotide 408]; SKOV3 [G*T*G→G*C*G (Val→Ala), nucleotide 969]; TT*A*→TT*G* (Leu→Leu), nucleotide 1030]. The cell line EG had weak maspin expression, detected by Western blot and immunohistochemistry, but no mRNA expression was detected by RT-PCR or Northern blot analysis. The cell line 222 had no detectable maspin by any of the modalities used. The tumor 1044.01 was obtained from a patient with stage III ovarian cancer and expressed maspin by Western blot, RT-PCR (Fig. 1, *A* and *D*), and immunohistochemistry (data not shown).

Wild-Type Maspin Suppresses Invasion in Ovarian Carcinoma. Wild-type maspin cDNA was transfected into 222 and SKOV3 ovarian cancer cells (Fig. 2). Immunofluorescence and immunohistochemical peroxidase staining demonstrated that most of the transfected maspin localized to the nucleus (Fig. 2). The baseline invasion rates of these ovarian cancer cell lines, measuring invasive ability through a basement membrane matrix *in vitro*, have been reported previously (17). SKOV3 is moderately invasive (6.3%), and the cell line 222 is highly invasive (11.8%). After maspin transfection, two stably transfected clones (222-MC2 and 222-MC3) were tested for *in vitro* invasive ability. Wild-type maspin decreased stimulated invasion rates by 67% (from 12.04% to 4%) in 222-MC2 and by 44% in 222-MC3 (Fig. 3). The cell line SKOV3 was success-

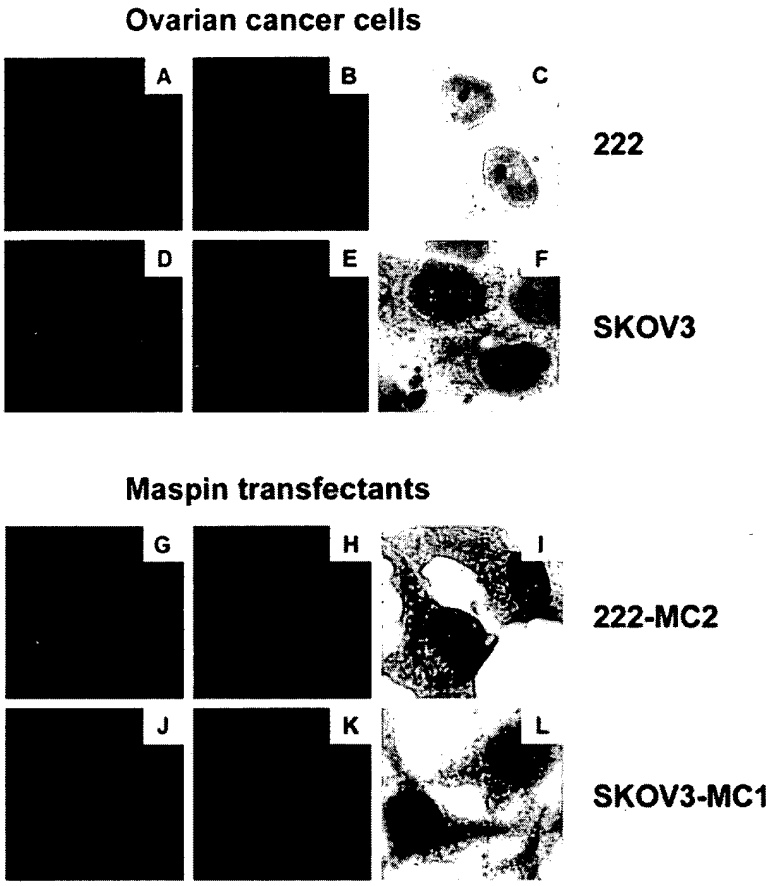

Fig. 2 Immunofluorescence and immunohistochemical peroxidase staining for *maspin* in the ovarian cancer cell lines 222 (*A–C*) and SKOV3 (*D–F*), and the maspin transfectants 222-MC2 (*G–I*) and SKOV3-MC1 (*J–L*). *A*, *D*, *G*, and *J* are negative controls. ×630.

fully transfected with wild-type maspin, and three stable clones (SKOV3-MC1, -MC2, and -MC3) were further tested. The average invasive activity of the maspin-transfected clones was decreased by 62–68% when compared with SKOV3. There was no significant difference in invasion of either cell line with sham transfection (Fig. 3*B*). To prove that the effect on invasion was indeed caused by wild-type maspin expression, a monoclonal antibody (2 μg/ml) was used to inhibit maspin activity. As shown in Fig. 4, the antibody had no significant effect on the invasive activity of the 222 cell line. However, when the cell line 222-MC2 was exposed to the antibody, the invasive ability was restored to baseline levels. In contrast, even although SKOV3 and OVCAR3 cells expressed maspin, the monoclonal antibody had no significant effect on their invasive ability *in vitro*.

Maspin Expression in Human Ovarian Samples. Maspin expression was assessed using immunohistochemistry in 14 benign epithelial ovarian tumors, 10 ovarian tumors of LMP, and 80 invasive epithelial ovarian cancers. Representative staining results in these samples are shown in Fig. 5. Maspin expression was seen in four benign epithelial ovarian tumors and was mostly nuclear in localization (Fig. 5*A*). All of the LMP tumors had maspin staining. More specifically, eight (80%) of LMP tumors had mostly nuclear staining (Fig. 5*B*), and only two tumors had cytoplasmic staining primarily. Among the invasive ovarian cancers, 57 (71%) were considered positive, based on the OMS of ≥ 1, and 23 (29%) were considered negative. Thirty (37%) of the invasive tumors overexpressed maspin (OMS = 3). Among the 57 invasive ovarian cancers with OMS ≥1, only 15 (26%) had ≥50% nuclear staining ($P = 0.001$ compared with benign and LMP tumors).

Clinicopathological Features of Invasive Ovarian Cancers. The demographic features of the patients in this study are listed in Table 1. The mean age of patients in this cohort was 59.6 years. Eighty-one % of all of the patients had advanced stage (III or IV) disease and 43% had high-grade (III) disease. Sixty-eight % of all of the patients underwent optimal surgical cytoreduction (less than 1 cm of residual disease at the end of surgery). The correlation of maspin overexpression and various clinical variables are listed in Table 2. There was no association of maspin overexpression with histological subtype (serous *versus* other), stage, likelihood of nodal positivity, or menopausal status. Maspin overexpression was associated with high tumor grade and presence of ascites. Thirty-eight (76%) of 50 patients with tumor OMS ≤2 achieved optimal cytoreduction, compared with only 53% of those with maspin overexpression ($P = 0.04$). As expected, survival was adversely affected by high-stage, high-grade, and residual disease >1 cm (all *P*s

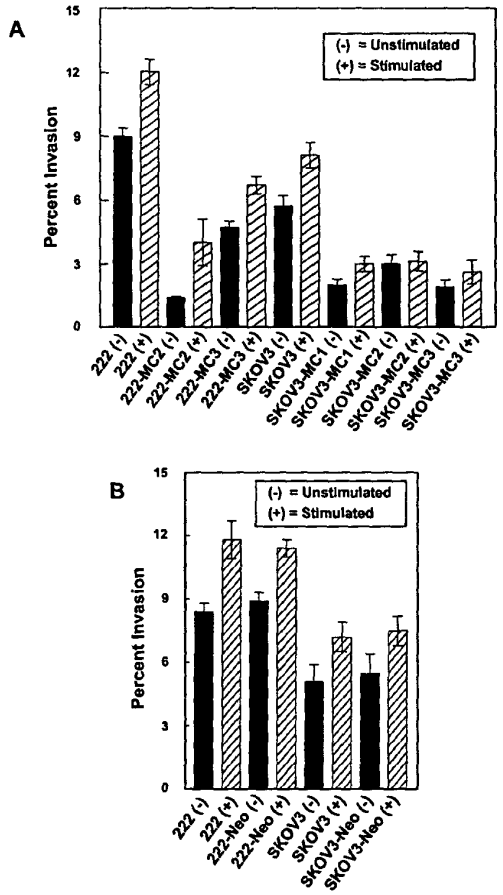

Fig. 3 Invasion profile of ovarian cancer cell lines (*222* and *SKOV3*) compared with (*A*) maspin transfectants (*222-MC2*, *222-MC3*, *SKOV3-MC1*, *SKOV3-MC2*, and *SKOV3-MC3*); and (*B*) sham transfectants (*222-Neo* and *SKOV3-Neo*), based on their ability to invade a basement membrane matrix *in vitro*, in the presence (▨) or absence (■) of a chemoattractant. *Error bars*, SE.

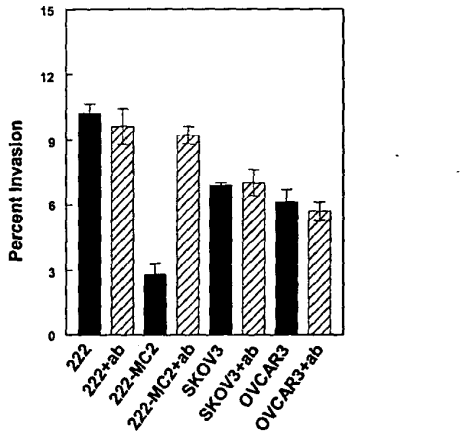

Fig. 4 Invasion profile of ovarian cancer cell lines, based on their ability to invade a basement membrane matrix *in vitro* with or without an inhibitory monoclonal antibody to maspin (*ab*). *Error bars*, SE.

<0.05, data not shown). In univariate analysis, maspin overexpression was associated with significantly worse survival (median survival, 6.33 *versus* 2.67 years; $P = 0.003$; Fig. 6). However, the presence of ≥50% nuclear maspin was associated with improved survival ($P = 0.003$). In multivariate analysis using the Cox proportional hazards model that involved age, stage, grade, volume of residual disease, maspin overexpression, and maspin subcellular localization, only high-stage ($P < 0.02$) and OMS of 3 ($P < 0.02$) were significant predictors of poor survival.

DISCUSSION

Maspin was originally described as a tumor suppressor gene important in cell motility and invasion (1). High *maspin* expression has been noted in normal human mammary and prostate epithelial cells but is decreased in the cancer cells from these respective organs, and is lost in metastatic cells (12, 13). Previously published data regarding *maspin* expression in normal ovarian tissue showed no expression in normal ovaries (11). However, maspin very likely plays a significant role in ovarian function, *e.g.*, the ovarian ovulation efficiency of maspin heterozygote mice is greatly reduced compared with controls.[4] The homozygous *maspin* knockout mice are known to be embryonic lethal. With respect to cancer progression, maspin inactivation that results in a lack of expression in prostate and breast tumors has been thought to play a role in tumor invasion and motility. Studies indicate that the loss of maspin expression during tumor progression results from both the absence of transactivation through the Ets element and the presence of transcription repression through the negative HRE recognized by the androgen receptor (19).

Interestingly, recent findings in pancreatic cancer are in sharp contrast to those reported in breast and prostate tumors. Specifically, Maass *et al.* (15) reported that maspin was detected in five of nine pancreatic cancer cell lines but was not detected in normal pancreatic tissue. In addition, maspin was expressed in 23 of 24 tumor specimens, but clinical correlates were not presented. Most of the tumor samples had strong cytoplasmic staining, but subcellular localization of *maspin* was not described in the preneoplastic pancreatic lesions. Similar to the pancreatic model, *maspin* was minimally expressed to absent in normal and benign ovarian samples in our present study. It is possible that the subcellular localization of maspin may play a critical role in its biological function. Alterations in subcellular localization and function of genes have been reported with other genes including β-*catenin* (20–22), *cyclin E* (23), *12-lipooxygenase* (24), the cyclin-dependent kinase inhibitor *p27(Kip1)* (25), and *cyclin D1* (26). Aust *et al.* (22) have demonstrated that, compared with normal colonic epithelium, colorectal cancers showed an overall shift from membranous to cytoplasmic expression of β-*catenin* and *E-cadherin*. Sgambato *et al.* (25) showed that, compared with normal colonic epithelium, the amount of *p27(Kip1)* in the cytoplasmic fraction was significantly higher in colon tumor samples. In our study, most of the

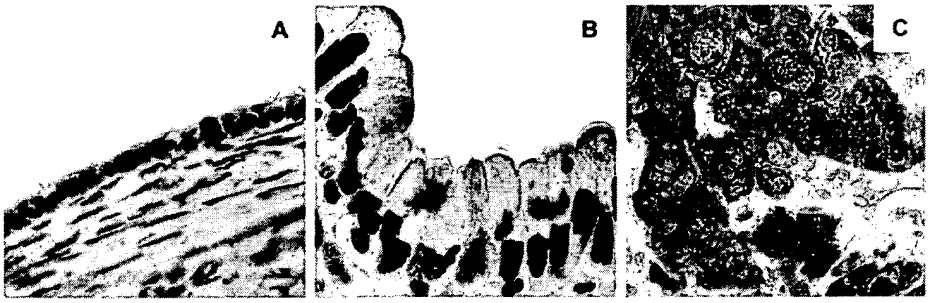

Fig. 5 Representative immunohistochemical peroxidase staining for maspin in: (*A*) normal ovary, (*B*) LMP ovarian tumor, and (*C*) stage IV, grade III ovarian cancer obtained from a patient. ×630.

Table 1 Demographic features of invasive ovarian cancer patients

Variable	*n*
Age, yr	59.6 (34–83)
Stage	
I	10
II	5
III	51
IV	14
Menopausal	
Yes	61
No	19
Histology	
Serous	52
Other	28
Grade	
Low (I or II)	46
High (III)	34
Ascites	
Yes	60
No	20
Cytoreduction	
Optimal	54
Suboptimal	26
Node status	
Positive	10
Negative	22
Not done	48
Status	
Alive without disease	21
Alive with disease	12
Dead of disease	43
Dead of other causes	4

Table 2 Correlation of clinicopathological variables with maspin overexpression in invasive ovarian cancer patients

	Maspin overexpression		
Variable	Yes (n = 30)	No (n = 50)	P
Stage			
Low	4	11	0.34
High	26	39	
Histology			
Serous	22	30	.23
Other	8	20	
Grade			
Low (I or II)	11	35	0.004
High (III)	19	15	
Ascites			
No	3	17	0.02
Yes	27	33	
Cytoreduction			
Optimal	16	38	0.04
Suboptimal	14	12	
Node status			
Positive	3	7	0.16
Negative	5	17	
Not done	22	26	
Menopausal			
Yes	5	14	0.25
No	25	36	

LMP tumors of the ovary expressed maspin, although it was mostly nuclear in localization. Analogous to invasive pancreatic tumors, the majority of invasive ovarian cancers also expressed cytoplasmic maspin staining. Thus, it is tempting to speculate that the maspin expressed in these tumors may be inactive. Indeed, our data with the SKOV3 cell line would support this finding: invasion was not affected by an inhibitory antibody. However, definitive proof for this hypothesis will require a functional assay of maspin activity, which is presently unavailable.

The *maspin* gene is part of a *serpin* locus at chromosome 18q21.3-q23. Several important transcription factor-binding sites are present within the 1-kb promoter region, namely Ets, AP1, HRE, and p53 (6). It contains seven exons (exon 1 is noncoding) and 6 introns. Interestingly, the region on 18q containing *maspin* is one of the most common areas of loss of heterozygosity in ovarian carcinoma (27). However, no significant mutations within the *maspin* coding sequence have been reported to date. Lack of any significant mutations in *maspin* is consistent with its being a class-I tumor suppressor molecule because such molecules are not expected to harbor any mutations within the coding sequences.[3] Although, at present, the molecular and biological mechanisms of the maspin function remain unknown, there is evidence that maspin interacts with the p53 tumor suppressor pathway and may function as an inhibitor of angiogenesis *in vitro* and *in vivo* (10, 12). Also, maspin appears to be regulated by wild-type p53. Zou *et al.* (28) reported that there was robust induction of maspin in prostate and breast cancer cells after wild-type p53 expression. p53 was found to activate the maspin promoter by binding directly to the p53 consensus binding site present in the maspin promoter. It is not known whether such reliance on wild-type p53 occurs in other tumor systems. Most ovarian cancers are known to harbor *p53* gene mutations (29, 30). Therefore, we are actively inves-

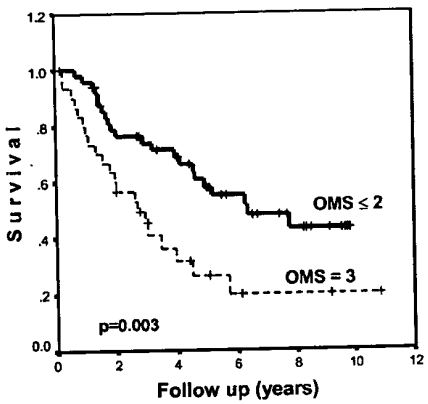

Fig. 6 Kaplan-Meier survival of ovarian cancer patients based on *maspin* staining intensity using the log-rank statistic.

tigating the role of p53 in maspin expression in ovarian carcinoma to address this possible relationship.

Ovarian cancer continues to result in a high mortality rate among gynecological malignancies. This is reflective of the advanced stage at presentation in most patients. There are no reliable preoperative tests that consistently distinguish pelvic masses into benign or malignant. Because cytoplasmic maspin was detected in most ovarian cancer patients and it is minimally expressed in benign adnexal masses, it may serve as a useful marker for the detection of ovarian cancer. Paradoxically, nuclear localization of maspin (which may represent the active form of this molecule) was associated with improved survival, which may represent an additional utility.

In summary, *maspin* was found to be minimally expressed in adult normal ovarian epithelium. This study provided the first evidence that maspin is expressed by a substantial proportion of ovarian cancers. We have also provided *in vitro* evidence that wild-type *maspin* can inhibit ovarian cancer invasion. The precise role of *maspin* in ovarian cancer remains to be demonstrated. However, it is clear that the nuclear localization of *maspin* is associated with increased survival, whereas the cytoplasmic localization is associated with poor outcome in ovarian carcinoma, thus rendering it an attractive therapeutic target.

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